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14. ABSTRACT

Traumatic eye injury (TEI) is one of the leading causes of monocular blindness in military personnel and young males worldwide. This profound and frequently irreversible posttraumatic loss of vision has a poor prognosis due to retinal cell death, scar formation, and lack of functional regeneration. Proliferative vitreoretinopathy (PVR), a form of intraocular fibrosis, is often the primary reason for the loss of vision after ocular trauma, and frequently occurs after blunt trauma and open globe injuries caused by penetration, rupture, perforation, and presence of intraocular foreign bodies as well as after retinal re-attachment surgery. We genetically engineered "protease activity sensor" (PAS) as chimeric transmembrane protein that can respond to increase in metalloproteinase activity by shedding/releasing tagged-ectodomains in the vicinity of affected cells after traumatic eye injury and induction of PVR. We demonstrated that upon infection with AAV carrying our construct, HEK293 cells and neurons in culture expressed the engineered HA-tagged PAS proteins. Their HA-tagged ectodomains were detected in the extracellular medium within minutes following stimulation with ionomycin and glutamate respectively. We used a rabbit PVR model of ocular trauma in which autologous blood was injected into the vitreous cavity of one eye after a surgical incision through the pars plana. We have successfully developed engineered adeno-associated virus (AAV) with CAG and CMV promoters, to transduce primary cortical neurons and retinal cells. These vectors will be applied in *vivo* to deliver therapeutic genes after ocular trauma.

15. SUBJECT TERMS
Trauma, Injury, Eye, Retina, PVR, Neuroinflammation, Proteases, Metalloproteinases, Cell death, Gene Therapy

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Table of Contents

Page

INTRODUCTION:

Traumatic eye injury (TEI) is one of the leading causes of monocular blindness in military personnel and young males worldwide (Negrel and Thylefors, 1998). This profound and frequently irreversible posttraumatic loss of vision has a poor prognosis due to retinal cell death, scar formation, and lack of functional regeneration. Proliferative vitreoretinopathy (PVR), a form of intraocular fibrosis, is often the primary reason for the loss of vision after ocular trauma, and frequently occurs after blunt trauma and open globe injuries caused by penetration, rupture, perforation, and presence of intraocular foreign bodies as well as after retinal re-attachment surgery (Mietz et al., 1994; Cardillo et al., 1997). Many of the pathological processes driving PVR can be traced to the local, inflammatory processes that trigger several downstream mechanisms including the activation of metalloproteinases (MPs), that facilitate extracellular matrix (ECM) remodeling, proliferation and migration of inflammatory cell, inducing formation and contraction of periretinal membranes resulting in PVR and retinal detachment (Symeonidis et al., 2011). Since the activation of MPs is an immediate event following the primary injury, the instantaneous and specific release of therapeutics at the site of injury may limit the extent of inflammation, provide sufficient cytoprotection and reduce the probability of PVR following the TEI.

1. KEYWORDS:

Trauma, Injury, Eye, Retina, PVR, Neuroinflammation, Proteases, Metalloproteinases, Cell death, Gene Therapy

3. ACCOMPLISHMENTS:

Major goals of the project

1. To obtain animal protocol approval from the local Animal Care Committee (ACC) at University of British Columbia (UBC), equivalent to the Institutional Animal Care and Use Committee (IACUC) in US, and then to obtain the regulatory approval for use of animal subjects from the Animal Care and Use Review Office (ACURO)
2. To evaluate the expression and activation of MPs and plasma membrane shedding in the eye after ocular trauma
3. To develop of transmembrane proteins that will respond to the increase of MPs activity in animal model of PVR
4. To evaluate two therapeutic-PAS (Protease Activity Sensors) in PVR animal model of ocular trauma

What was accomplished under these goals?

We have initiated the work using the experimental animal model of ocular trauma to evaluate the expression and activity of metalloproteinases in the eye after ocular trauma as potential biomarkers of injury. We have evaluated the rabbit model of PVR, as an experimental model of ocular trauma that was used to evaluate our trauma-induced gene therapy and its delivery after ocular injury.

To initiate the work on the animal model we have developed animal protocol "Mechanisms of Ocular Disease" IACUC protocol number A14-0320 and received regulatory approvals by the Animal Care Committee (ACC) at the University of British Columbia (UBC), a Canadian equivalent

to IACUC, and subsequently by ACURO at DoD. In addition we have obtained regulatory approvals from IACUC and ACURO for our amended protocol for the use of AAV viruses to deliver in the eye gene therapy designed to alleviate the effects of ocular trauma.

Significant results or key outcomes

We genetically engineered "protease activity sensor" (PAS) as chimeric transmembrane protein that can respond to increase in metalloproteinase activity by shedding/releasing tagged-ectodomains in the vicinity of affected cells after traumatic eye injury and induction of PVR.
These membrane spanning PAS construct consists of 2 reporter elements, including green fluorescent protein (GFP) (Tsien, 1998), and a hemagglutinin A (HA) tags (Zhao et al., 2013). A protease cleavage site, engineered to be sensitive to injury/inflammation activated membrane associated proteases of the ADAM and MMP families (Candelario-Jalil et al., 2009; Rosenberg, 2009) is located just extracellular to the membrane spanning domain of the construct. In the earlier reports we demonstrated that upon infection with AAV carrying our construct, HEK293 cells in culture expressed the engineered HA-tagged PAS proteins. The HA-tagged ectodomains from our engineered constructs were detected in the extracellular medium within minutes following stimulation with ionomycin.

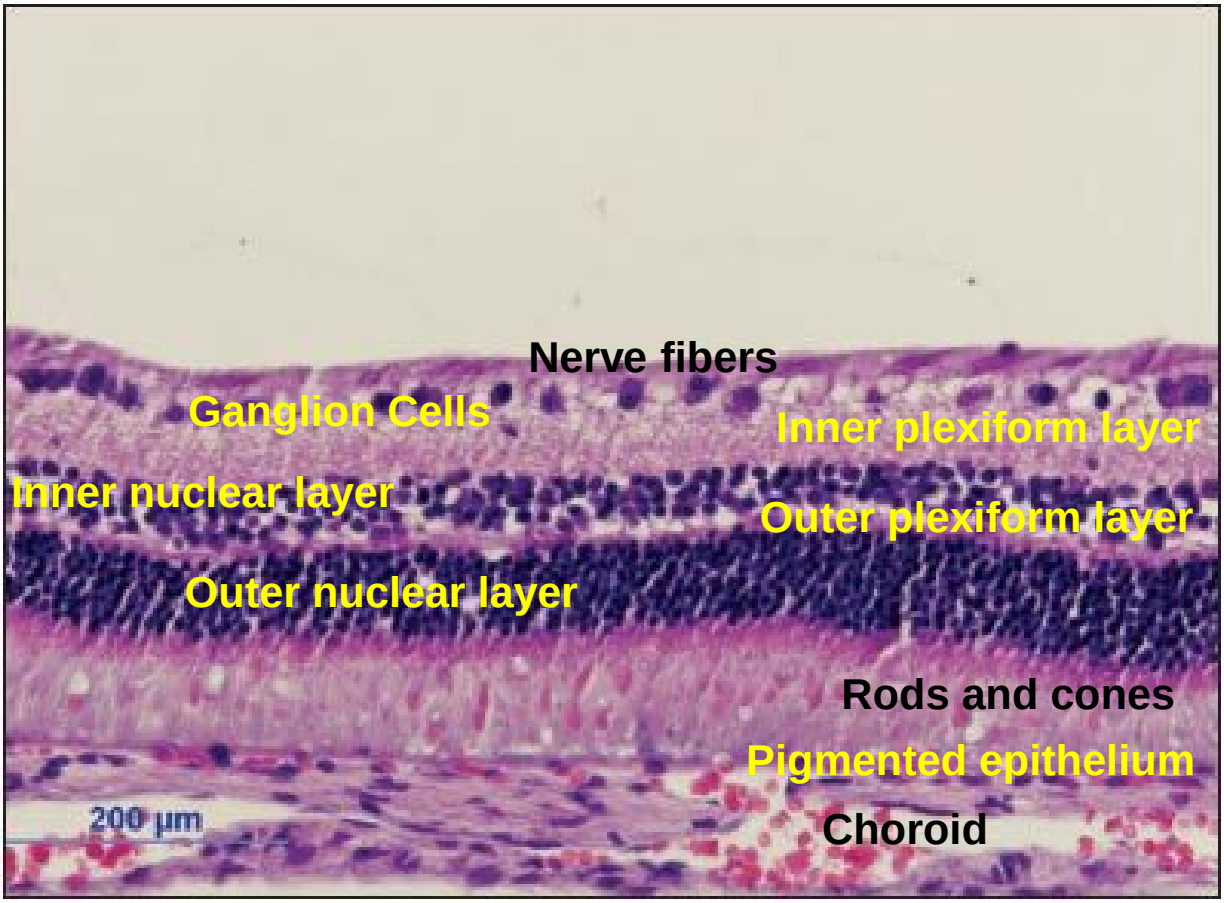
PVR as an Experimental model of open globe ocular trauma

We have initiated *in vivo* studies to evaluate PVR as an experimental model of open globe ocular trauma (Agrawal et al., 2007) that will be used for testing and optimization of our gene therapy for ocular trauma *in vivo*.

Accumulation of blood in the vitreous due to hemorrhage is the most important independent predictive factor for PVR following blunt and open globe ocular trauma. To simulate a trauma model of PVR, one of the eyes in rabbits was injured by a standard 8 mm circumferential scleral incision through the pars plana 2.5 mm from the limbus to the center of the vitreous cavity avoiding the lens and the peripheral retina. The prolapsed vitreous was abscised and the wound was closed with 8-0 silk sutures. At the end of the surgery, all eyes will receive 0.4ml of autologous blood, drawn immediately before the incision, injected through a 25-gauge needle inserted through the wound and into the mid vitreous under ophthalmoscopic control. These animals need to be followed for several months because the complete retinal detachment induced by PVR typically occurs after 6-8 weeks. The contralateral eye remained untouched to avoid any confounding effects, and also served as a contralateral control. The retinal status was evaluated with retinal imaging. Eyes were enucleated for histological and biochemistry analysis, IHC and WB to assess the extent retinal injury and its mechanisms including the expression and activation of metalloproteinases in the eye and the membrane shedding events after ocular trauma.

We have initiated *in vivo* studies to evaluate the expression and activation of metalloproteinases in the eye after ocular trauma using an experimental model of rabbit PVR (Agrawal et al., 2007) including the eye expression of metalloproteinases ADAM10, ADAM17, MMP2, and MMP9, after ocular trauma. In rabbit animal model we have followed their expression during the evolution of the injury (2-17 weeks).

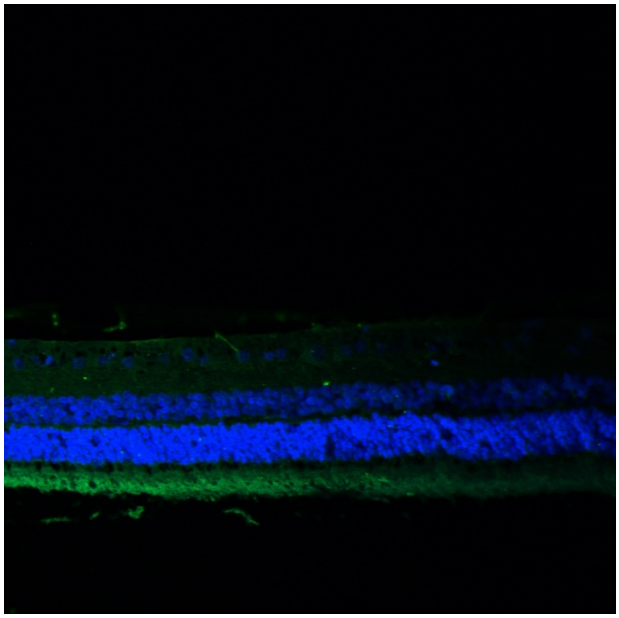
Diagram of normal retinal histology in rabbit eye



The diagram of normal retinal histology of rabbit eye is provided as a visual guidance for the IHC data that is presented in this report.

ADAM10 immunofluorescence - 2 weeks after ocular trauma-induced PVR

A



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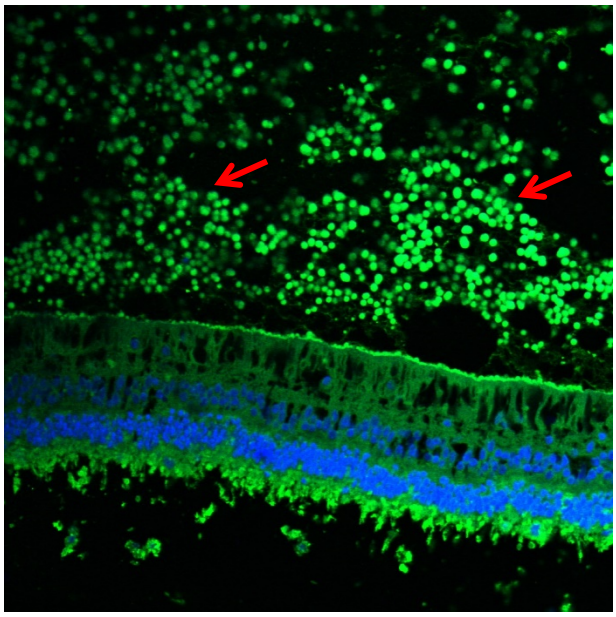


Figure 1. A. Control eye with saline injection. No membrane and ADAM10 immunopositive staining is seen in the control eye. **B.** Experimental eye at 2 weeks after a penetrating injury with blood injection. There is a loss of cells in the inner layers of the neuroretina, and an increased ADAM10-immunopositivity in the outer layers (photoreceptors). No epiretinal membranes were present at 2 weeks. Injected blood cells showed strong autofluorescence in the vitreous (red arrows). (20X; blue-DAPI nuclear staining; green-ADAM10 immunofluorescence).

ADAM10 immunofluorescence - 12 weeks after ocular trauma-induced PVR

A **B**

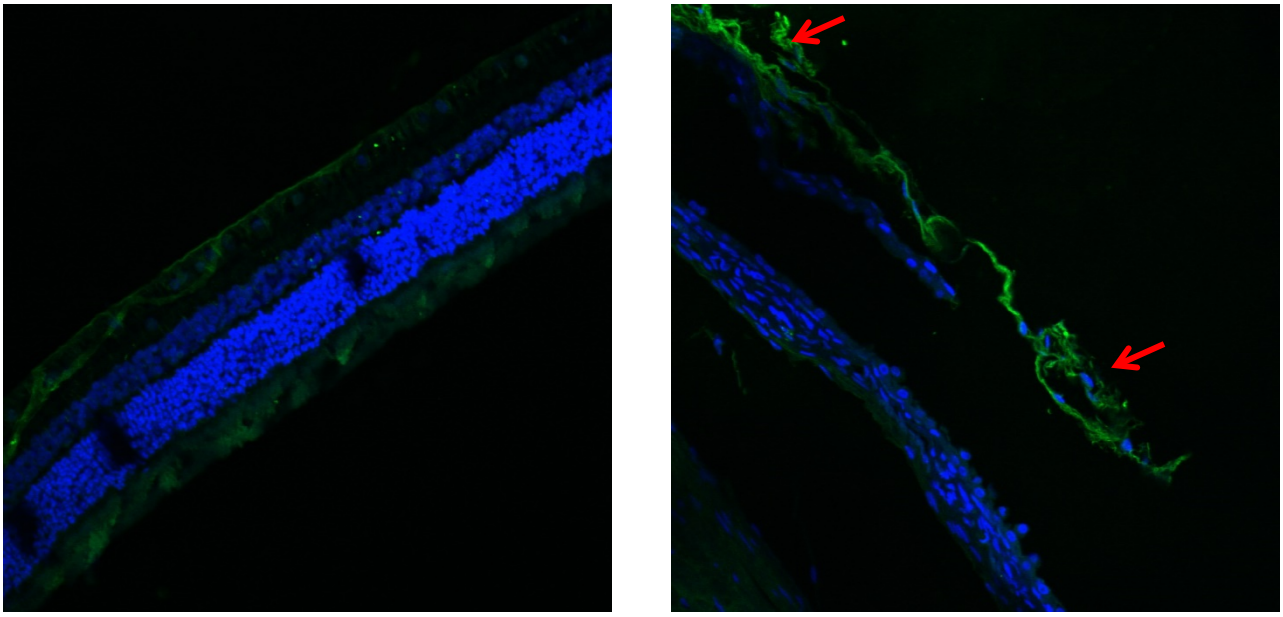


Figure 2. A. Control eye with saline injection. No membrane and ADAM10 immunopositive staining is seen in the control eye. **B.** Experimental eye at 12 weeks after a penetrating injury with blood injection. Epiretinal membranes in the vitreous (arrows) and total retinal detachment were observed in addition to ADAM10 immunopositive staining of the epiretinal membranes. (20x; blue-DAPI nuclear staining; green-ADAM10 immunofluorescence).

ADAM17 immunofluorescence - 2 weeks after ocular trauma-induced PVR

A **B**

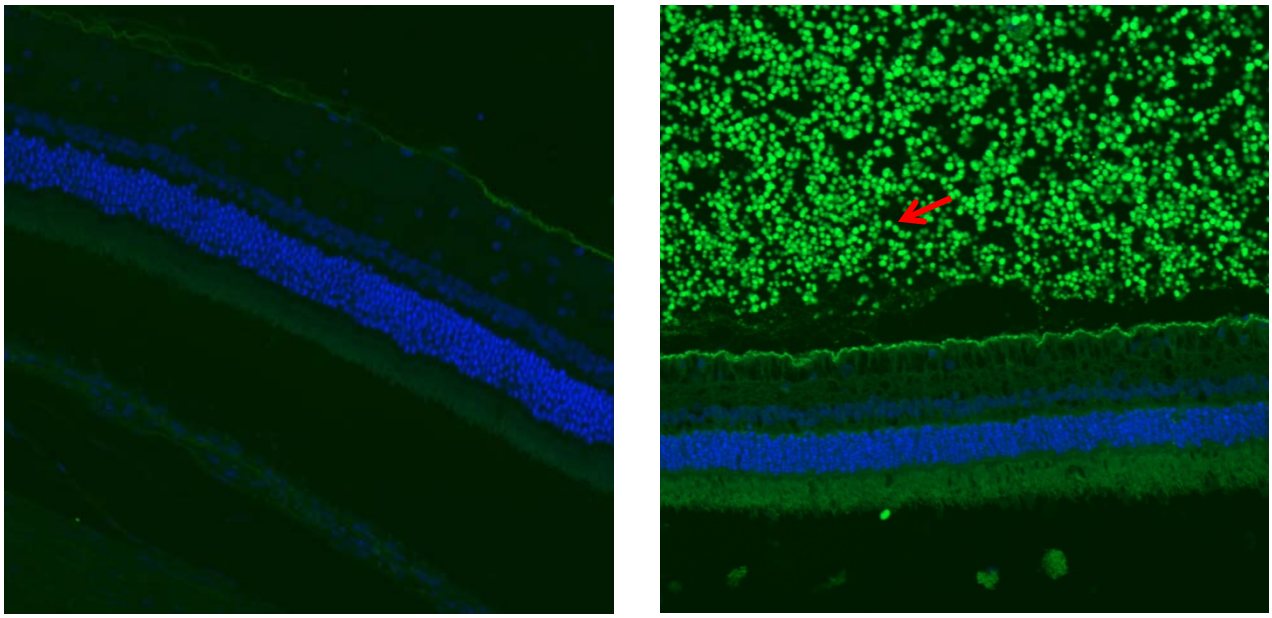
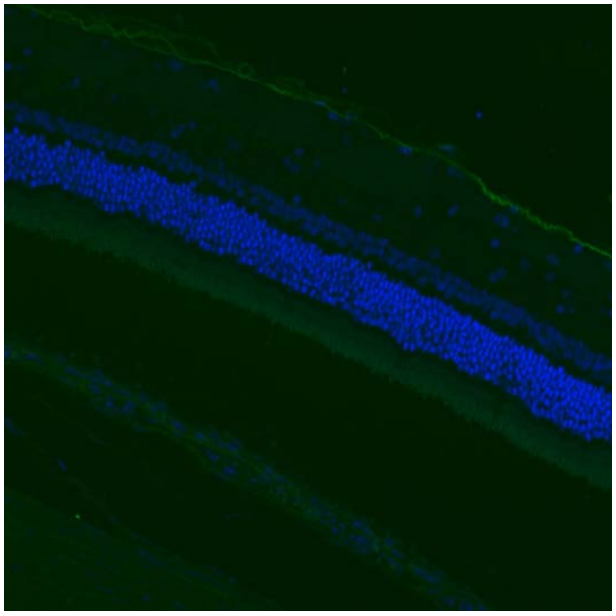


Figure 3. A. Control eye with saline injection. No membrane and ADAM17 immunopositive staining is seen in the control eye. B. Experimental eye at 2 weeks after a penetrating injury with blood injection. There is a loss of cells in the inner layers of the neuroretina, and an increased ADAM17-immunopositivity in the outer layers (photoreceptors). No epiretinal membranes were present at 2 weeks. Injected blood cells showed strong autofluorescence in the vitreous (red arrow). (20X; blue-DAPI nuclear staining; green-ADAM17 immunofluorescence).

ADAM17 immunofluorescence - 12 weeks after ocular trauma-induced PVR

A



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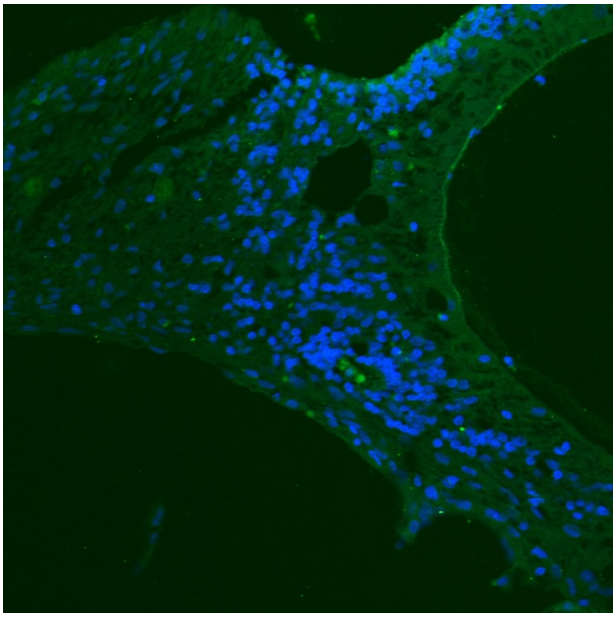

C

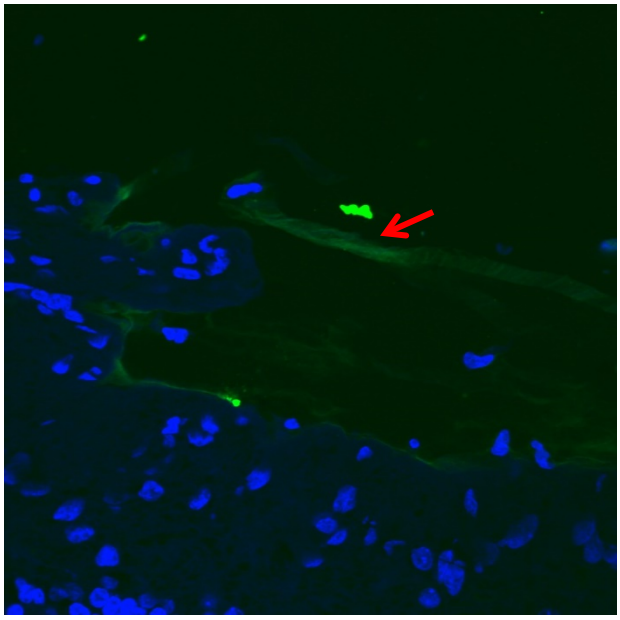

Figure 4. A. Control eye with saline injection. No membrane and ADAM17 immunopositive staining is seen in the control eye. **B.** Experimental eye 12 weeks after penetrating injury with blood injection. Epiretinal membranes in the vitreous and total retinal detachment were observed in addition to ADAM17 immunopositive staining of the epiretinal membranes (20X). **C.** The same experimental eye 12 weeks after the ocular injury as in B. Epiretinal membranes (red arrow) in the vitreous with ADAM17 immunopositive staining at higher magnification (40X). (blue-DAPI nuclear staining; green-ADAM17 immunofluorescence).

Bright field images of rabbit eye 17 weeks after ocular trauma

A **B**

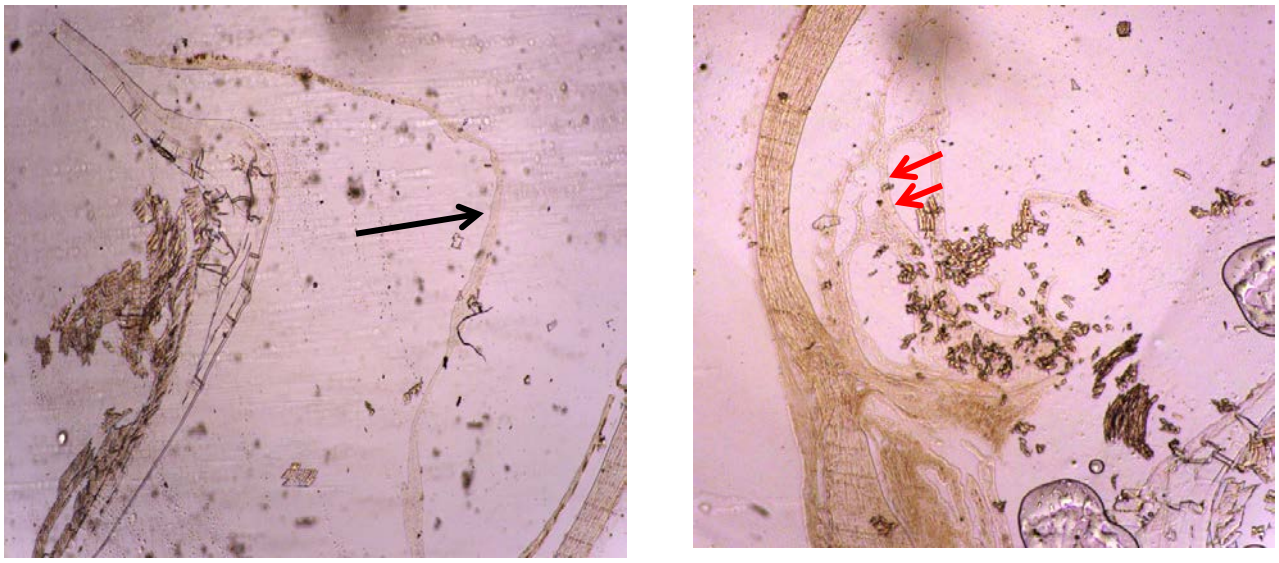

Figure 5. Bright field images of rabbit eye 17 weeks after the penetrating injury followed with blood injection. The proliferation and migration of inflammatory cells induce formation and contraction of epiretinal membranes resulting in PVR and retinal detachment **A.** The ocular trauma induced PVR with retinal detachment. The black arrow points to the fully detached retina. **B.** The formation of epiretinal membranes is indicated with red arrows.

The stages of Rabbit PVR Pathogenesis:

Stage 1: vitreous haze, intravitreal and /or epiretinal membrane (ERM)
Stage 2: focal traction, localized vascular changes (membrane-induced)
Stage 3: localized detachments or pucker (membrane–induced)
Stage 4: extensive retinal detachment (membrane-induced)
Stage 5: total retinal detachment, fold, and/or formation of a hole (membrane-induced)

Summary:

1. The blood induction of rabbit PVR at 2 weeks reached stage 1

2. The blood induction of rabbit PVR at 12 weeks reached stage 5

3. Both ADAM10 and ADAM17 showed weak staining of the PVR membrane at at 12 weeks (stage 5)

4. Blood in vitreous cavity showed strong autofluorescence (green in Fig 1B and Fig. 3B)

MMP2 immunofluorescence - 2 weeks after ocular trauma-induced PVR

A **B**

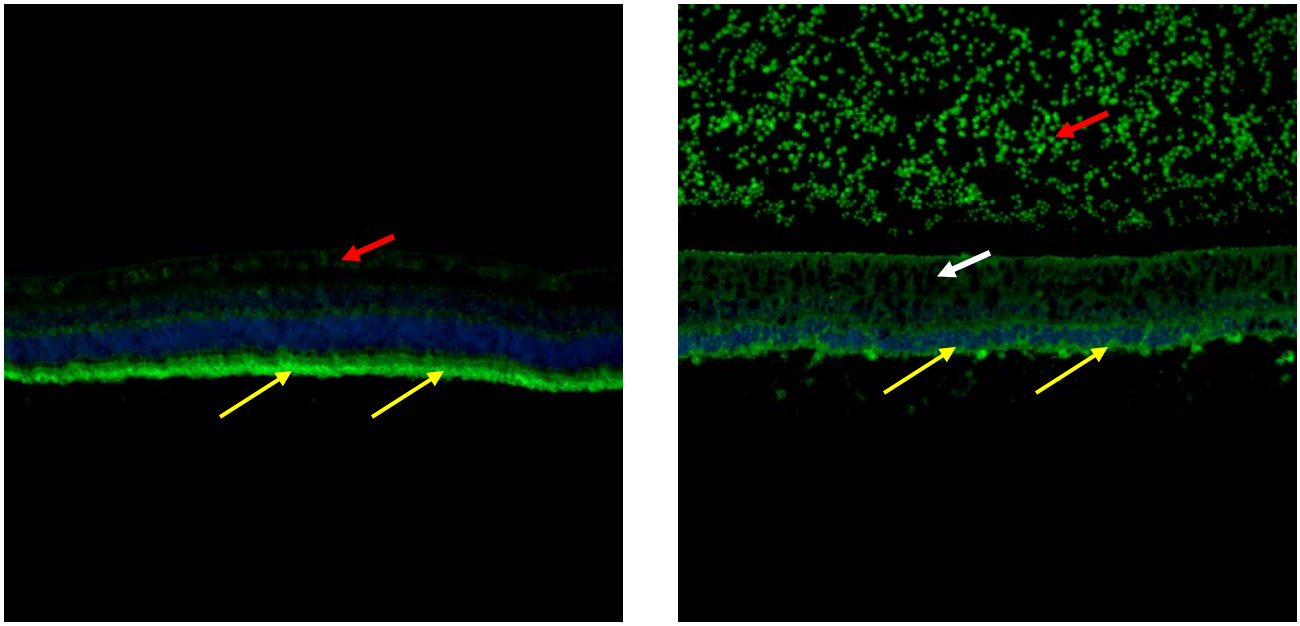

Figure 6. A. Control rabbit eye after control saline injection. MMP2 immunostaining is seen in the inner layer (ganglion cell layer) of the control eye (red arrow). The inner plexiform layer is negative for MMP2 in the control eye. There is a significant autofluorescence in the outer segments of the retina, present in the absence of the primary MMP2 antibody (yellow arrows). Healthy photoreceptor outer segments (yellow arrows) can be seen in the control retina. No epiretinal membranes were present in the control eye. **B.** Experimental eye at 2 weeks after a penetrating injury and autologous blood injection has lost the basic cytoarchitectonics. Injected blood cells showed strong autofluorescence in the vitreous (red arrow). There is a loss of cells in the inner layers of the neuroretina, with an increase of MMP2-immunopositivity (white arrow). The experimental eye shows degenerated photoreceptors outer segments (yellow arrows). No epiretinal membranes were present at 2 weeks. (20X; blue=DAPI nuclear staining; green=MMP2 immunofluorescence).

MMP2 immunofluorescence - 12 weeks after ocular trauma-induced PVR

A **B**

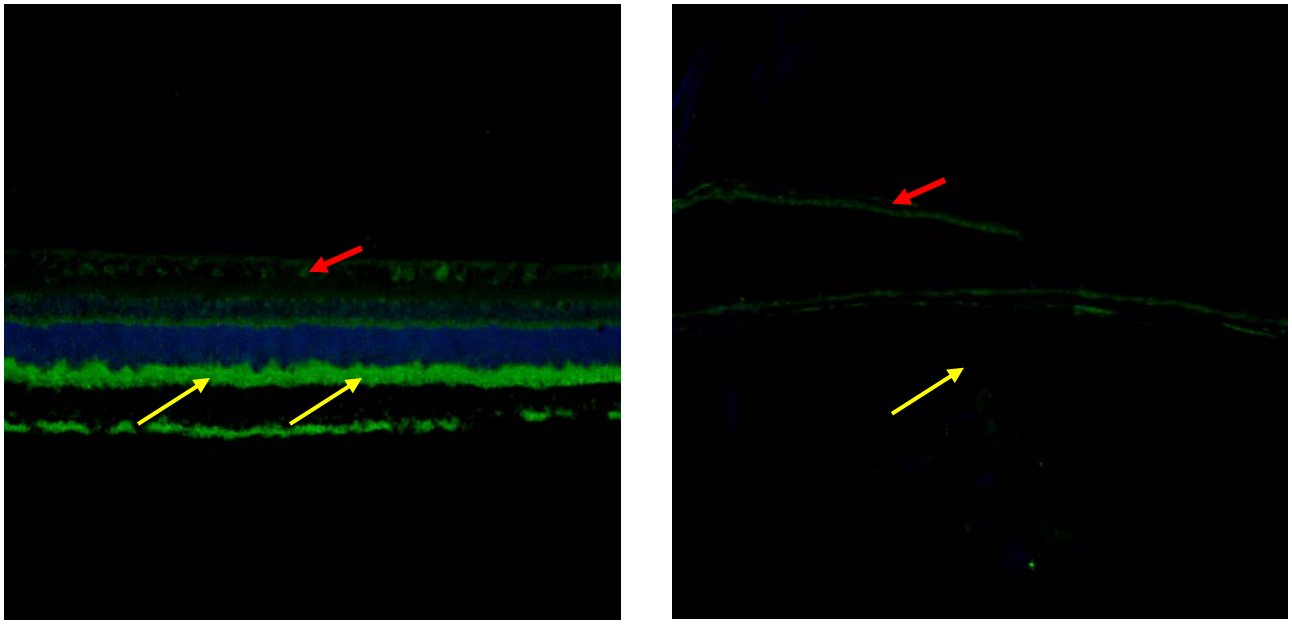

Figure 7. A. Control rabbit eye after control saline injection. MMP2 immunostaining is seen in the inner layer (ganglion cell layer) of the control eye (red arrow). The inner plexiform layer is negative for MMP2 in the control eye. There is a significant autofluorescence in the outer segments of the retina, present in the absence of the primary MMP2 antibody (yellow arrows). Healthy photoreceptor outer segments (yellow arrows) can be seen in the control retina. No epiretinal membranes were present in the control eye. **B.** Experimental eye at 12 weeks after a penetrating injury and autologous blood injection. Epiretinal membranes in the vitreous (red arrow) and total retinal detachment (yellow arrow) were observed in addition to the low level MMP2 immunostaining of the epiretinal membranes. (20x; blue=DAPI nuclear staining; green=MMP2 immuno-fluorescence).

MMP9 immunofluorescence - 2 weeks after ocular trauma-induced PVR

A **B**

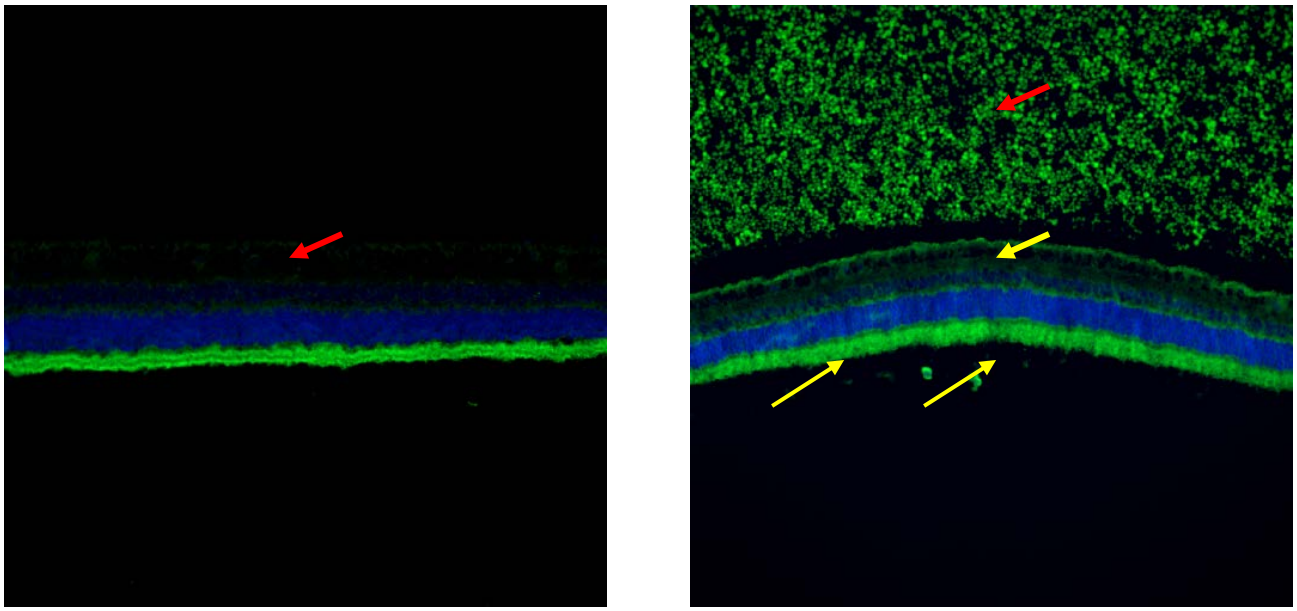

Figure 8. A. Control rabbit eye after control saline injection. No epiretinal membrane and low MMP9 immunopositive staining is seen in the control eye (red arrow). The inner plexiform layer is negative for MMP9 in the control eye. There is a significant autofluorescence in the outer segments of the retina, present in the absence of the primary MMP9 antibody (yellow arrows). Healthy photoreceptor outer segments (yellow arrows) can be seen in the control retina. **B**. Experimental eye at 2 weeks after a penetrating injury and autologous blood injection. Injected blood cells showed strong autofluorescence in the vitreous (red arrow). There is a considerable loss of cells in the inner layers of the neuroretina, with an increased MMP9 immunostaining (yellow arrow). No epiretinal membranes were present at 2 weeks. (20X; blue=DAPI nuclear staining; green=MMP9 immunofluorescence).

MMP9 immunofluorescence - 12 weeks after ocular trauma-induced PVR

A **B**

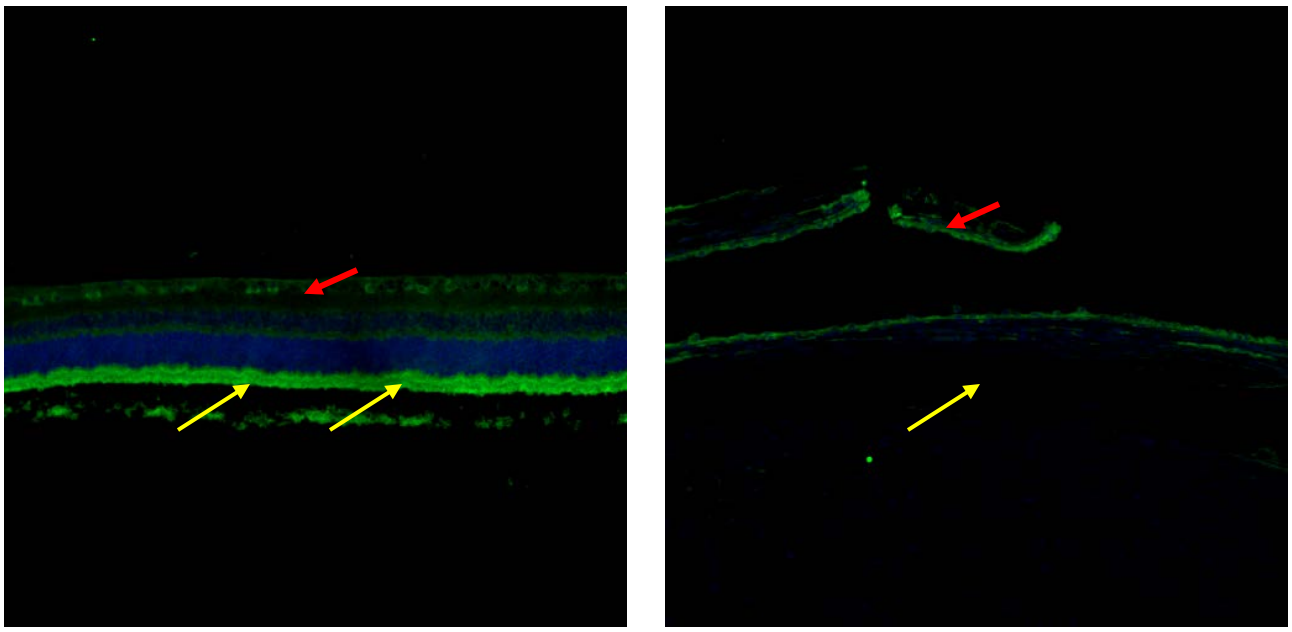

Figure 9. A. Control rabbit eye after control saline injection. Low level MMP9 immunostaining is seen in the inner layers of the control eye (red arrow). The inner plexiform layer is negative for MMP9 in the control eye. There is a significant autofluorescence in the outer segments of the retina that is present in the absence of the primary MMP9 antibody (yellow arrows). Healthy photoreceptor outer segments (yellow arrows) can be seen in the control retina. **B.** Experimental eye at 12 weeks after a penetrating injury and autologous blood injection. Epiretinal membranes in the vitreous (red arrow) and total retinal detachment (yellow arrow) were observed in addition to the low level MMP9 immunostaining of the epiretinal membranes (red arrow). (20x; blue=DAPI nuclear staining; green=MMP2 immuno-fluorescence).

Immunohistochemistry staining of epiretinal membranes (ERM) 4 weeks after ocular trauma

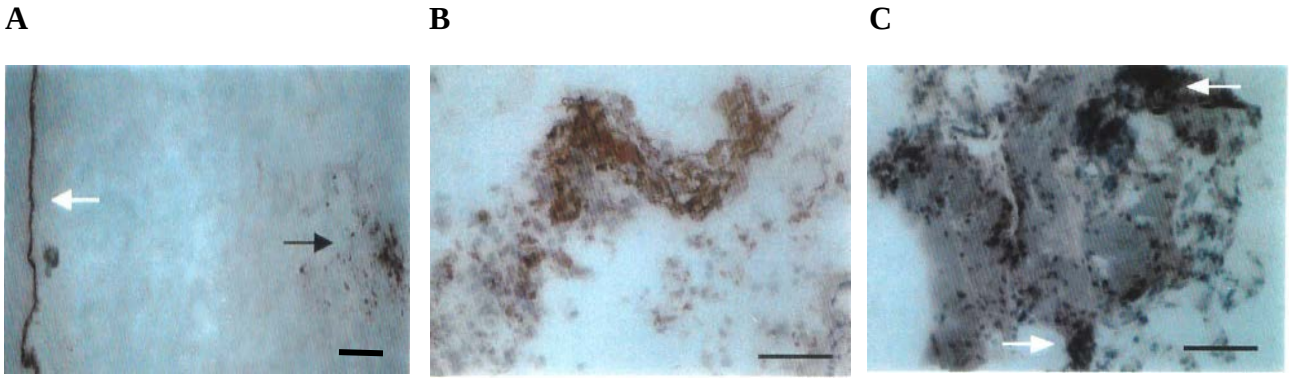

Figure 10. Immunohistochemistry staining of epiretinal membranes (ERM) in rabbit eye 4 weeks after ocular trauma (stage 3 PVR). **A**. A positive immunoreactivity for cytokeratin of the RPE monolayer (white arrow), and the RPE cells throughout the ERM (black arrows) was observed. **B**. Positive immunoreactivity of the ERM for alpha smooth muscle actin (αSMA) and **(C)** for glial fibrillary acidic protein (GFAP) indicates a presence of myofibroblasts and glial cells respectively, that are dominant cell types in the ERMs. Scale bars: (A-C) 25 µm.

Summary:

1. The blood induction of rabbit PVR at 2 weeks reached stage 1

2. Blood in vitreous cavity showed strong autofluorescence (green in Fig 6B and Fig. 8B)

3. The blood and injury-induced PVR in rabbit eye reached stage 5 at 12 weeks, with total retinal detachment (Fig. 7B and Fig. 9B)

4. Immunohistochemistry for both MMP2 and MMP9 showed a weak staining of the ERM at 12 weeks post injury

5. Epiretinal membranes (ERMs) are present in the vitreous 12 weeks after the eye injury that are immunoreactive for alpha smooth muscle actin (αSMA) and glial fibrillary acidic protein (GFAP) indicating the presence of myofibroblasts and glial cells respectively, the dominant type of cells in the ERMs (Fig. 10)

We have reported earlier that that carry our PAS constructs under the control of the Ubiquitin promoter can successfully deliver and expressed PAS in HEK-293 cells and primary neuronal cultures. However, the same plasmids and AAV vectors with the Ubiquitin promoter were not able deliver and express PAS in retinal pigment epithelium (RPE) cells (ARPE19 cell line) that are one of the target cells in the proposed ocular trauma therapy.

Promoter sequences are vital for proper transgene transduction in the retina (Dinculescu et al., 2005). The ubiquitously expressed cytomegalovirus (CMV) enhancer promoter (Grant et al., 1997; Green et al., 2001), chicken β-actin promoter (Pang et al., 2006), the fusion of the chicken β-actin promoter and CMV (Sawicki et al., 1998), and ubiquitin promoter (Zhou and Dean, 2007) were demonstrated to provide stable and constitutive reporter expression of various transgenes in the eye.

To successfully continue this project we have decided to additionally evaluate other promoters that can drive genes in the eye. We have initiated studies that will initially test the CMV, and later the 'CMV early enhancer/chicken β actin' (CAG) hybrid promoter with established capacity to deliver gene therapy in the eye.

We report here that the CMV promoter in the CMV-PAS-GFP plasmid did drive the expression of our PAS therapeutic constructs in HEK-293, cultured neurons, and the RPE cells (ARPE19) *in vitro*.

However, plasmids are not the best tool for delivery of therapeutic transgenes *in vivo*, as they cannot provide long-term expression that is necessary for achieving the desired therapeutic effects. To facilitate the *in vivo* transgene delivery we have developed a new AAV2-CMV-PAS-GFP viral vector that was successfully tested *in vitro* in HEK-293, neurons and glia cells (Fig 11-13 and Fig. 15).

Surprisingly, the same **AAV2-CMV-PAS-GFP vector was not able to transduce the RPE cells** *in vitro*, that are the cells directly involved in the pathophysiology of PVR, retinal detachment and loss of vision after eye injury.

HEK-293 are infected with AAV2-CMV-PAS-GFP Vector

A **B**

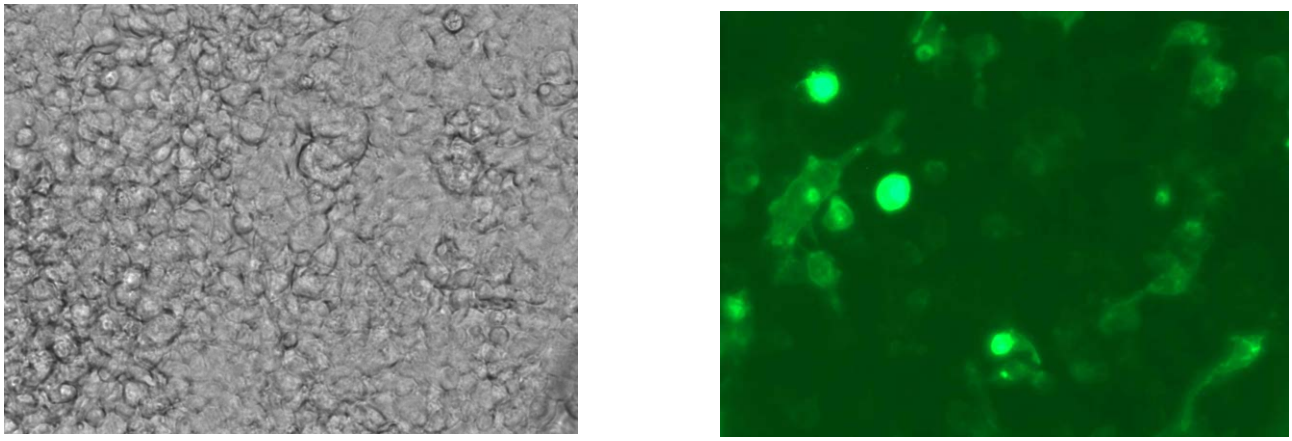

Figure 11. GFP-tagged PAS transgene was transduced with the AAV2-CMV-PAS-GFP viral vector in HEK-293 Cells at 4 x 10^{10} vg/mL. **A.** Bright field images of the infected HEK-293 cells. **B.** The presence of GFP-tagged constructs in the cytoplasm and plasma membrane was confirmed with GFP fluorescence microscopy. (20X; green=GFP fluorescence).

Cultured Neurons are infected with AAV2-CMV-PAS-GFP Vector

A **B**

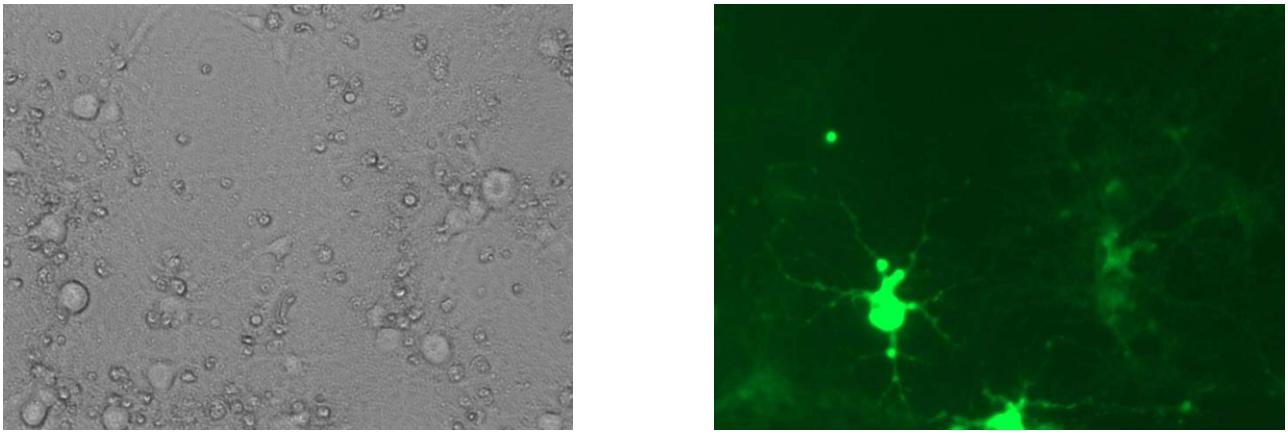

Figure 12. GFP-tagged PAS transgene was transduced with the AAV2-CMV-PAS-GFP viral vector in culture of primary neurons at 4 x 10^{10} vg/mL. **A.** Bright field images of the infected neurons. **B.** The presence of GFP-tagged constructs in the cytoplasm and plasma membrane was confirmed with GFP fluorescence microscopy. (20X; green=GFP fluorescence).

Glia cells are infected with AAV2-CMV-PAS-GFP Vector

A **B**

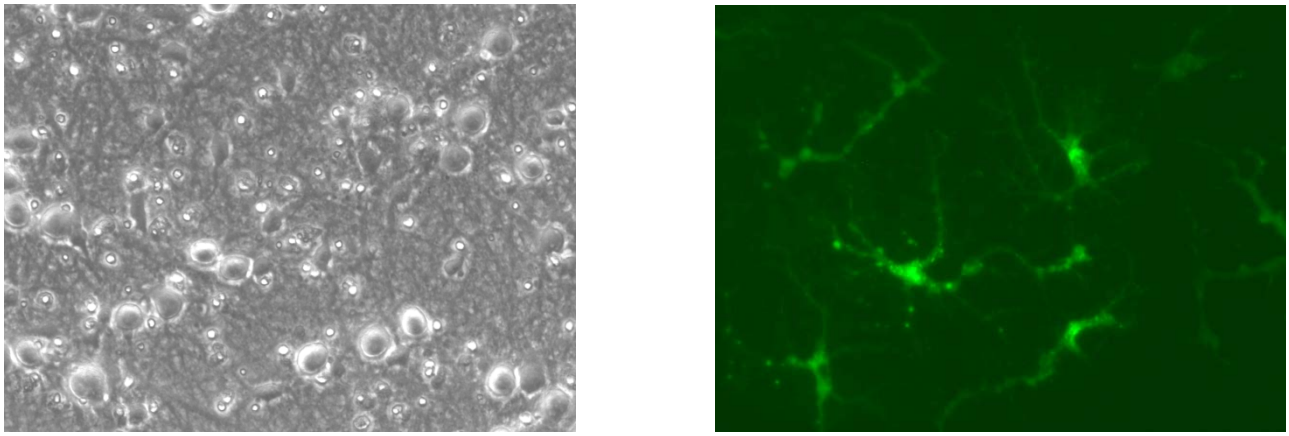

Figure 13. GFP-tagged PAS transgene was transduced with the AAV2-CMV-PAS-GFP (at 4 x 10^{10} vg/mL) viral vector in primary cortical culture. **A.** Bright field images of the infected glia cells *in vitro*. **B.** The presence of GFP-tagged constructs in the cytoplasm and plasma membrane was confirmed with GFP fluorescence microscopy. (20X; green=GFP fluorescence).

ARPE19 cells are Transfected with CMV-PAS-GFP plasmid

A **B**

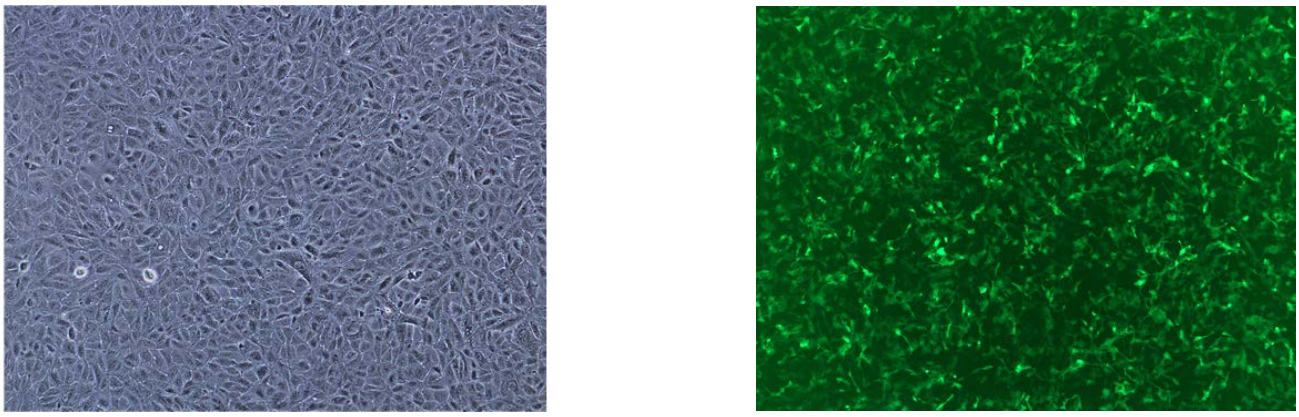

Figure 14. ARPE19 cells were seeded in a 12-well culture plate and incubated with the CMV-PAS-GFP plasmid (1µg/well) for 48h. Transfection efficiency was evaluated with fluorescent microscopy 48h post transfection. **A.** Bright field images of transfected ARPE19 cells *in vitro*. **B.** GFP-tagged constructs were detected in the cytoplasm and plasma membrane of ARPE19 cells using GFP fluorescence microscopy. (4X; green=GFP fluorescence).

ARPE19 cells are NOT infected with AAV2-CMV-PAS-GFP Vector

A **B**

Figure 15. ARPE19 cells were seeded in a 12-well culture plate and incubated with the AAV2-CMV-PAS-GFP viral vector (at 2 x 10^{10} - 4 x 10^{11} vg/mL) for 48-96h. Transduction efficiency was evaluated with fluorescent microscopy 48 and 96 hours post infection. **A.** Bright field images of the AAV2 infected ARPE19 cells *in vitro*. **B.** GFP-tagged constructs were NOT detected in the cytoplasm and plasma membrane of ARPE19 cells using GFP fluorescence microscopy. (4X; green=GFP fluorescence).

Summary:

1. In the earlier reports we demonstrated that plasmids based on the ubiquitin promoter can successfully deliver our therapeutic PAS-GFP transgene in HEK-293 and neurons, but NOT in retinal cells (ARPE19) *in vitro.*

2. The CMV promoter based plasmids were able to successfully deliver our therapeutic PAS-GFP transgene in HEK-293, neurons, as well as in ARPE19 cells *in vitro* (Fig 14). However, plasmids are not the best tool for delivery of therapeutic transgenes *in vivo,* and they cannot provide long-term transgene expression that is necessary for achieving the desired therapeutic effect.

3. To facilitate the *in vivo* transgene delivery we created a new AAV2-CMV-PAS-GFP viral vector that was tested *in vitro* (Fig 11-13 and Fig. 15).

4. HEK-293 cell line (Fig. 11), neurons (Fig. 12) and glia cells (Fig. 13) from primary cortical cultures were successfully transduced with our AAV2-CMV-PAS-GFP viral vector.

5. Surprisingly, the same AAV2-CMV-PAS-GFP viral vector was not able to transduce the RPE cells *in vitro*, retinal cells that are involved in the pathophysiology of PVR, retinal detachment and loss of vision, serious sequelae of ocular trauma.

Cultured Neurons are infected with AAV9-CAG-GFP control vector

A **B**

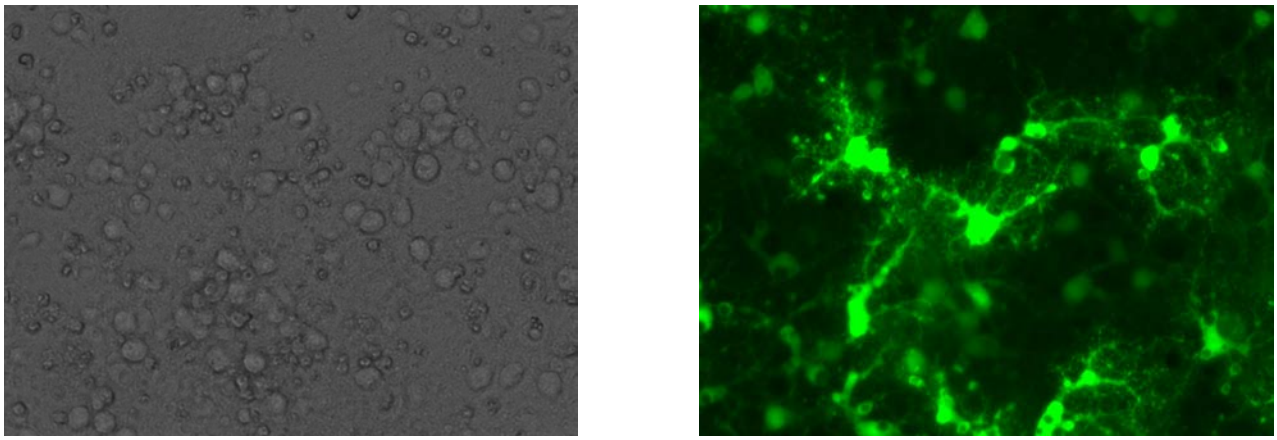

Figure 16. We used engineered adeno-associated virus (AAV) with CAG promoter AAV9-CAG-GFP to transduce primary cortical culture (10^{10} vg/mL). The presence of GFP in the cytoplasm was confirmed with GFP fluorescence microscopy. (20X; green=GFP fluorescence).. **A.** Bright field images of the infected neurons. **B.** The presence of GFP-tagged constructs in the cytoplasm and plasma membrane was confirmed with GFP fluorescence microscopy. (20X; green=GFP fluorescence).

Cultured Neurons are infected with AAV2-CMV-GFP control vector

A **B**

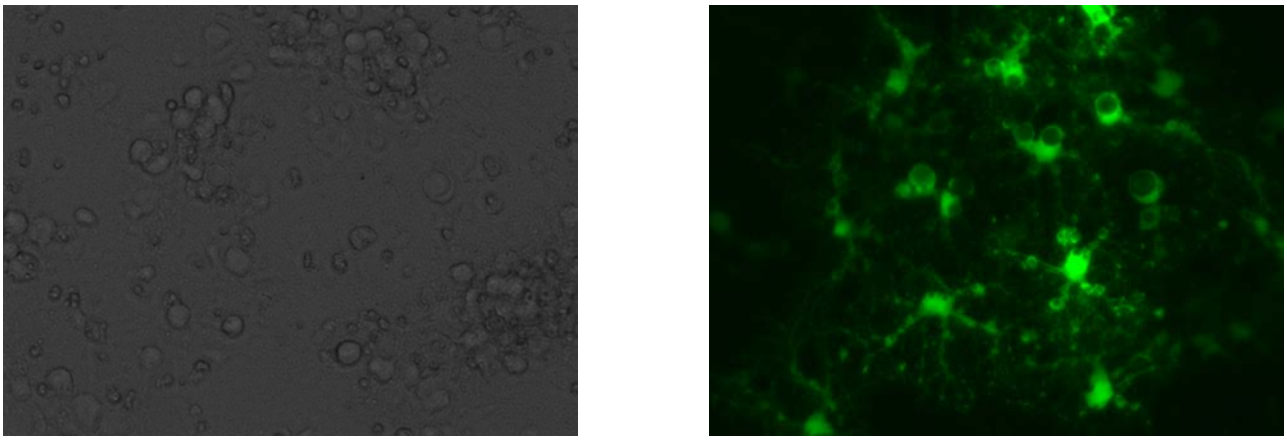

Figure 17. We used engineered AAV2-CMV-GFP with CMV promoter to transduce primary cortical culture (10^{10} vg/mL). The presence of GFP in the cytoplasm was confirmed with GFP fluorescence microscopy. (20X; green=GFP fluorescence). **A.** Bright field images of the infected neurons. **B.** The presence of GFP-tagged constructs in the cytoplasm and plasma membrane was confirmed with GFP fluorescence microscopy. (20X; green=GFP fluorescence).

What opportunities for training and professional development has the project provided?
Nothing to Report.

How were the results disseminated to communities of interest?
Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

During the 12 months extension we expect to achieve the following grant objectives:

1. Develop new AAV vectors (AAV9 and AAV2) that can deliver transgenes in the eye, targeting primarily the RPE cells but also glial and or endothelial cells. The transgenes expression will be driven by two promoters (CMV and CAG) to provide long-term and robust expression of therapeutic genes in the injured eye.
2. Test the new AAV serotypes and promoters in RPE cells *in vitro*.
3. Evaluate the expression and distribution of the therapeutic transgenes after subretinal delivery in rabbit eye, using the AAV vectors and promoters selected *in vitro*.
4. Evaluate the therapeutic potential of these therapeutic tools in rabbit model of PVR as an experimental model of eye trauma.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Promoter sequences are vital for proper transgene transduction in the retina (Dinculescu et al., 2005). We have reported earlier that plasmids that carry our PAS constructs under the control of the Ubiquitin promoter can successfully deliver and expressed PAS in HEK-293 cells and primary neuronal cultures. However, the same plasmids and AAV vectors with the Ubiquitin promoter were not able deliver and express PAS in retinal pigment epithelium (RPE) cells (ARPE19 cell line) that are one of the target cells in the proposed ocular trauma therapy.

To proceed with the project successfully we initiated the evaluation of additional promoters that can drive transgenes in the eye such as the cytomegalovirus (CMV) enhancer promoter. Subsequently we reported that the CMV promoter in the CMV-PAS-GFP plasmid did drive the expression of our PAS therapeutic constructs in HEK-293, cultured neurons, and the RPE cells (ARPE19) *in vitro*. Furthermore, we found that AAV viral vectors, that we have developed based on the CMV-PAS-GFP plasmid, were successful in our *in vitro* studies using cultures of HEK-293 and cortical neurons.
Unfortunately, we found that the AAV viral vectors based on the CMV-PAS-GFP plasmid were not able to transduce RPE cells with our therapeutic Protease Activity Sensors driven by either human ubiquitin or CMV promoter. RPE are the cells that are directly involved in the pathophysiology of PVR, retinal detachment and loss of vision after eye injury.

We also plan to initiate studies that will evaluate the 'CMV early enhancer/chicken β actin' (CAG) hybrid promoter that also has an established capacity to deliver gene therapy in the eye. We have successfully tested the AAV9-CAG-GFP and AAV2-CMV-GFP control vectors in primary neuronal cultures (Fig. 16 and Fig. 17 respectively).

Additionally, to optimize the in vivo delivery of our PAS transgenes in the eye we will employ two viral serotypes (AAV9 and AAV2) shown to be able to deliver genes in the eye tissues.

Actual or anticipated problems or delays and actions or plans to resolve them
We expect that the volume of work needed to test two new promoters (CMV and CAG) and two additional viral serotypes (AAV9 and AAV2) to optimize the delivery of our PAS gene therapy in the eye would require 12 months extension of the expiration date of the award. This change would not involve any change in the approved objectives or scope of the project, and would not require any additional funding.

We have notified our USAMRAA Grants Officer two months prior to the expiration date of the award and requested one year extension of the performance period.

During the 12 months extension we expect to achieve the following grant objectives:

1. Develop new AAV vectors (AAV9 and AAV2) that can deliver transgenes in the eye, targeting primarily the RPE cells but also glial and or endothelial cells. The transgenes expression will be driven by two promoters (CMV and CAG) to provide long-term and robust expression of therapeutic genes in the injured eye.
2. Test the new AAV serotypes and promoters in RPE cells *in vitro*.
3. Evaluate the expression and distribution of the therapeutic transgenes after subretinal delivery in rabbit eye, using the AAV vectors and promoters selected *in vitro*.
4. Evaluate the therapeutic potential of these therapeutic tools in rabbit model of PVR as an experimental model of eye trauma.

Changes that had a significant impact on expenditures
Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report

6. PRODUCTS:

Publications, conference papers, and presentations
Nothing to Report

Website(s) or other Internet site(s)
Nothing to Report

Technologies or techniques
Nothing to Report

Inventions, patent applications, and/or licenses
Nothing to Report

Other Products
Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

PDs/PIs on the project

Name:	**Dr. William Jia**
Project Role:	PD/PI
Researcher Identifier (eRA ID):	WILLJIA
Nearest person month worked:	2
Contribution to Project:	Dr. Jia was coordinating the preparation and submission of required regulatory documents, the development of PAS constructs and their evaluation *in vitro*.

Name:	**Dr. Max Cynader**
Project Role:	Co-PD/PI
Researcher Identifier (eRA ID):	MAXCYNADER
Nearest person month worked:	1
Contribution to Project:	Dr. Cynader was reviewing the required regulatory documents; he was overseeing the *in vitro* experiments used for testing the PAS constructs.

Name:	**Dr. Joanne Matsubara**
Project Role:	Co-PD/PI
Researcher Identifier (eRA ID):	JOANNEMATSUBARA
Nearest person month worked:	1
Contribution to Project:	Dr. Matsubara was directly involved in the preparation and review of the required regulatory documents and is coordinating the *in vivo* experiments modeling ocular trauma.

Name:	**Dr. Ljubomir Kojic**
Project Role:	Co-Investigator
Researcher Identifier (eRA ID):	LJUBOMIRKOJIC
Nearest person month worked:	3
Contribution to Project:	Dr. Kojic was directly involved in the designing of the PAS constructs; he was preparing the animal protocol and other regulatory documents, including the quarterly and yearly reports.

Name:	**Dr. Jing Cui**
Project Role:	Co-Investigator
Researcher Identifier (eRA ID):	JINGCUI
Nearest person month worked:	3
Contribution to Project:	Dr. Cui was directly overseeing and conducting the *in vivo* studies; she was involved in developing of the animal protocol and preparing of the quarterly and yearly reports.

Name:	**Luke Bu**
Project Role:	Research Assistant
Researcher Identifier (eRA ID):	XUEXIANBU
Nearest person month worked:	2
Contribution to Project:	Mr. Bu was involved in the development and testing of the AAV vectors and PAS constructs *in vitro*, the preparing of the animal protocol and the quarterly and yearly reports.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
No other partner organizations were involved.

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHARTS:

We have submitted regularly the project Quad Charts as attachments with the Quarterly Technical Reports, and this Annual Technical Report.

9. APPENDICES:

10. References:

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Dinculescu A, Glushakova L, Min SH, Hauswirth WW (2005) Adeno-associated virus-vectored gene therapy for retinal disease. Human gene therapy 16:649-663.

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Symeonidis C, Papakonstantinou E, Souliou E, Karakiulakis G, Dimitrakos SA, Diza E (2011) Correlation of matrix metalloproteinase levels with the grade of proliferative vitreoretinopathy in the subretinal fluid and vitreous during rhegmatogenous retinal detachment. Acta Ophthalmol 89:339-345.

Tsien RY (1998) The green fluorescent protein. Annu Rev Biochem 67:509-544.

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Smart, Injury-triggered Therapy for Ocular Trauma

MR130584

PI: Dr. William Jia **Org:** University of British Columbia **Award Amount:** $249,600

Study/Product Aim(s)

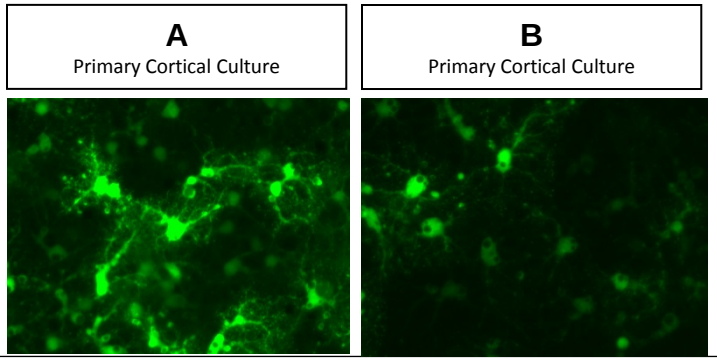
- To identify the metalloproteinases activated after ocular trauma.
- To develop and optimize genetically engineered trans-membrane proteins for instantaneous release of ectodomains in the vicinity of eye injury. To implement and optimize eye delivery methods using viral delivery strategies.
- To develop, optimize and validate "protease activity sensors" (PAS) able to deploy anti-inflammatory and neuroprotective "therapeutic ectodomains" (TE) in experimental models of PVR.

Approach

Our objective is to develop event-triggered gene therapy for PVR.

We propose to engineer PAS able to locally release therapeutic ectodomains by metalloproteinases activated in PVR, to provide neuroprotection and prevent loss of vision. Our "smart therapeutics" will exploit the activation metalloproteinases to provide instantaneous and specific neuroprotection immediately after ocular traumatic injury, prevent PVR and avert loss of vision.

In vitro delivery of PAS transgene in primary cortical cultures and ARPE19 cells



(A) We used engineered adeno-associated virus (AAV) with CAG promoter AAV9-CAG-GFP to transduce primary cortical culture (10^10 vg/mL). The presence of GFP in the cytoplasm was confirmed with GFP fluorescence microscopy. (20X; green=GFP fluorescence). **(B)** We used engineered AAV2-CMV-GFP with CMV promoter to transduce primary cortical culture (10^10 vg/mL). The presence of GFP in the cytoplasm was confirmed with GFP fluorescence microscopy. (20X; green=GFP fluorescence).

Timeline and Cost

Activities / CY	13	14	15	16
To obtain regulatory approval for animal model of ocular trauma and to identify metalloproteinases involved in eye injury				
To develop engineered transmembrane proteins that are shed and released in PVR and deliver them to the eye				
To evaluate and validate PAS for treatment of PVR after ocular trauma.				
Estimated Budget ($K)			**$125**	**$124.6**

Updated: (Oct 14, 2016)

Goals/Milestones

CY14-15 Goals – To obtain local IACUC and ACURO approval for in vivo protocol that addresses animal model for ocular trauma

CY15-16 Goals – To identify the metalloproteinases activated after ocular trauma and to develop PAS and eye gene delivery vector

☐Identify metalloproteinases activated after ocular injury using animal model of PVR

☐Implement and optimize viral delivery system for the eye

☐Develop PAS that are trauma-cleaved using *in vitro* and *in vivo* PVR models

CY17 Goals – To develop PAS carrying therapeutic domains (PAS-TE)

☐ Validate AAV-PAS and AAV-PAS-TE expression, and their *in vivo* therapeutic efficacy using experimental PVR model of ocular trauma.

Comments/Challenges/Issues/Concerns

- To test two new promoters (CMV and CAG) and two additional viral serotypes (AAV9 and AAV2) to optimize eye delivery of PAS gene therapy in the 12 months extension without funds.

Budget Expenditure to date

Projected Expenditure:$249,600 Actual Expenditure: $ 249,600